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Junction Adhesion Molecule Is a Receptor for Reovirus

Erik S. Barton,*‡# J. Craig Forrest,*‡# Jodi L. Connolly,*‡ James D. Chappell,†‡ Yuan Liu,§ Frederick J. Schnell,§ Asma Nusrat,§ Charles A. Parkos,§ and Terence S. Dermody*†‡‖
*Departments of Microbiology and Immunology
†Department of Pediatrics
‡Elizabeth B. Lamb Center for Pediatric Research
Vanderbilt University School of Medicine
Nashville, Tennessee 37232
§Division of Gastrointestinal Pathology
Department of Pathology and Laboratory Medicine
Emory University School of Medicine
Atlanta, Georgia 30322

Summary

Virus attachment to cells plays an essential role in viral tropism and disease. Reovirus serotypes 1 and 3 differ in the capacity to target distinct cell types in the murine nervous system and in the efficiency to induce apoptosis. The binding of viral attachment protein σ1 to unidentified receptors controls these phenotypes. We used expression cloning to identify junction adhesion molecule (JAM), an integral tight junction protein, as a reovirus receptor. JAM binds directly to σ1 and permits reovirus infection of nonpermissive cells. Ligation of JAM is required for reovirus-induced activation of NF-κB and apoptosis. Thus, reovirus interaction with cell-surface receptors is a critical determinant of both cell-type specific tropism and virus-induced intracellular signaling events that culminate in cell death.

Introduction

Mammalian reoviruses were one of the first animal virus systems to permit a molecular genetic approach to analysis of viral replication and pathogenesis. Reoviruses infect most children and can cause mild gastrointestinal or respiratory illnesses (Tyler and Fields, 1996). Studies of reovirus disease in mice established the importance of the viral attachment step as a critical determinant of disease outcome in the host (Sharpe and Fields, 1985). Following oral inoculation into newborn mice, serotype 1 (T1) reoviruses spread hematogenously to the CNS and replicate in ependymal cells, resulting in hydrocephalus. In contrast, serotype 3 (T3) reoviruses spread neurally to the CNS, where they replicate in neurons causing lethal encephalitis (Weiner et al., 1980). Since reovirus contains a segmented genome, pathogenic phenotypes can be ascribed to specific viral genes by screening reassortant viruses. Using this approach, it was determined that the mode of spread in the host (Tyler et al., 1986) and cell tropism in the CNS (Weiner et al., 1980) segregate with the viral S1 gene, which encodes the viral attachment protein, σ1.

Attachment protein σ1 forms an elongated fiber topped with a globular head (Fraser et al., 1990). The σ1 protein binds two types of cellular receptors using independent receptor-binding domains (RBDs). A domain in the fibrous tail of T3 σ1 binds α-linked sialic acid (SA) (Chappell et al., 1997, 2000). T1 σ1 also binds a cell-surface carbohydrate, but this molecule has not been defined (Chappell et al., 2000). A second RBD is located in the globular head of both T1 and T3 σ1. Recognition of the σ1 head receptor is important for virus binding and infection in vitro (Nagata et al., 1987; Turner et al., 1992; Nibert et al., 1995) and modulates CNS tropism in mice (Spriggs et al., 1983; Kaye et al., 1986). These data have led to a model in which the interaction of the σ1 head with distinct T1 and T3 receptors determines route of spread, tissue tropism, and resultant disease.

Despite the foundational nature of these observations for our understanding of mammalian reovirus tropism and pathogenesis, cellular receptors for the σ1 head domain have not been identified. Previous attempts to identify reovirus receptors have yielded promising candidates (Noseworthy et al., 1983; Co et al., 1985); however, the molecular cloning of cellular molecules conferring reovirus infection has not been reported. These earlier studies were perhaps confounded by the use of SA-binding T3 reovirus as an affinity ligand, an approach that might isolate highly sialyllated molecules regardless of their affinity for the σ1 head (Choi et al., 1990).

In addition to targeting viral replication to neurons, T3 σ1 triggers signaling events that lead to apoptosis of infected cells. Reovirus infection induces apoptosis in cultured cells (Tyler et al., 1995) and in vivo (Oberhaus et al., 1997). T3 reovirus strains induce apoptosis more efficiently than T1 strains, which is a phenotype regulated by the σ1-encoding S1 gene (Tyler et al., 1995). Reovirus infection also activates nuclear factor kappa B (NF-κB) (Connolly et al., 2000), an evolutionarily conserved transcription factor that plays critical roles in determining cell fate and regulating immune responses (Neurath et al., 1998; Barkett and Gilmore, 1999). Activation of NF-κB by reovirus is required for apoptosis induction, since apoptosis elicited by reovirus is significantly reduced in cells expressing a transdominant inhibitor of NF-κB and in cells deficient in the expression of NF-κB subunits p50 and p65 (Connolly et al., 2000). Together, these findings suggest that the binding of T3 σ1 to its receptors results in nuclear translocation of NF-κB and expression of cellular genes required for activation of the apoptotic machinery.

To permit dissection of the mechanism by which σ1–receptor interactions control reovirus tropism, apoptosis, and disease, we employed an expression-cloning approach to identify the cellular receptor for the T3 σ1 head domain. Screening of a human neuronal-precursor cell cDNA library identified junction adhesion molecule (JAM) as a reovirus receptor. JAM is a member of the immunoglobulin superfamily (IgSF) involved in regula-

‖To whom correspondence should be addressed (e-mail: terry.dermody@mcmail.vanderbilt.edu).
#These authors contributed equally to this work.

tion of tight junction (TJ) formation (Liu et al., 2000) and leukocyte transmigration across endothelium (Martin-Padura et al., 1998; Del Maschio et al., 1999). We demonstrate that non-SA-binding T1 and T3 reovirus strains are completely dependent on σ1–JAM interactions to establish infection. However, SA-binding strains are also capable of infection by a JAM-independent, SA-mediated pathway. NF-κB activation and apoptosis induced by SA-binding T3 reovirus requires interaction between T3 σ1 and JAM, indicating that reovirus infection is not sufficient to trigger apoptosis in the absence of JAM-induced signaling. These data indicate that JAM serves as a serotype-independent reovirus receptor capable of mediating virus attachment, infection, and intracellular signaling. Furthermore, by linking an integral TJ protein to the NF-κB signaling pathway, our results highlight a potential role for the TJ in regulating tissue-specific inflammatory responses to viral infection.

Results

Expression Cloning of hJAM, a Reovirus Receptor

To facilitate the identification of reovirus receptors based on the capacity to bind directly to the σ1 head, non-SA-binding T3 reovirus strain T3SA$^-$ was used as an affinity ligand (Barton et al., 2001) in a FACS-based expression-cloning approach (Aruffo and Seed, 1987). NT2 cells are human neuronal-precursor cells that support T3 reovirus infection (data not shown). An NT2 cDNA library was selectively enriched for cDNAs that confer binding of fluoresceinated T3SA$^-$ (FITC-T3SA$^-$) virions to transfected COS-7 cells (Figures 1A–1D). After four rounds of FACS enrichment and sib selection, four clones were identified that conferred FITC-T3SA$^-$ binding to greater than 20% of transfected cells, which was equivalent to the transfection efficiency in these experiments (Figure 1D). All four clones encoded hJAM (Liu et al., 2000), which suggests that hJAM is a reovirus receptor.

Antibodies Directed against hJAM Inhibit Reovirus Infection by Abrogation of Virus Binding

To test the hypothesis that hJAM is a reovirus receptor, we determined whether anti-hJAM monoclonal antibodies (mAbs) could inhibit reovirus infection (Figure 2). For these experiments, we used NT2 cells (Figure 2A), HeLa cells (Figure 2B), and Caco-2 cells (Figures 2C and 2D), an intestinal epithelial cell line. Cells were treated with anti-hJAM mAbs 7G2C9 or J10.4 (Liu et al., 2000) prior to infection with T3SA$^-$. In the absence of anti-hJAM antibodies, T3SA$^-$ grew efficiently in all three cell types. In contrast, anti-hJAM mAbs dramatically inhibited T3SA$^-$ growth, resulting in 10- to 100-fold reduction in viral yield. In each case, decreased viral yield was mediated by reduction in the number of infected cells (Figure 2D and data not shown). These results indicate that hJAM binding is critical for reovirus infection of multiple cell types, including cells of intestinal and neural lineages.

To determine whether anti-hJAM mAbs inhibit reovirus infection at the viral attachment step, we assessed the capacity of these mAbs to block binding of radiolabeled T3SA$^-$ and an SA-binding T3 strain T3SA$^+$ (Barton et al., 2001) to NT2 cells (Figure 2E). Binding of T3SA$^-$ to NT2 cells was inhibited by unlabeled competitor virus,

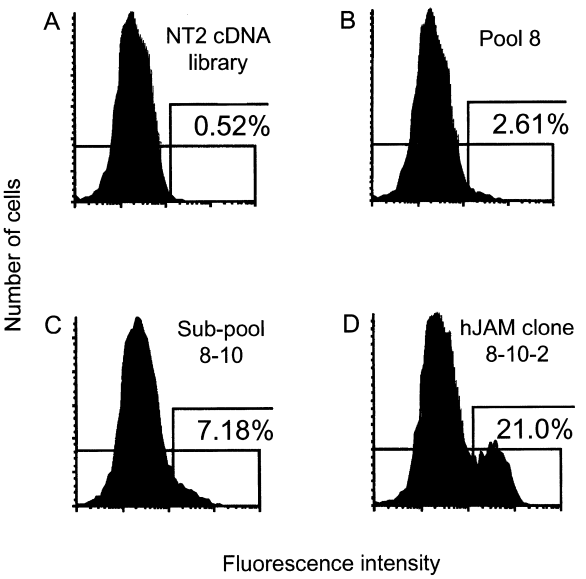

Figure 1. Identification of JAM as a Reovirus Receptor by Expression Cloning

(A) COS-7 cells were transfected with an NT2-cell cDNA library and incubated with FITC-labeled T3SA$^-$ virions. The 0.5% most fluorescent cells were collected by FACS for plasmid rescue. Percent cells binding virus in (B), (C), and (D) is expressed relative to cells transfected with NT2-cell cDNA library.

(B) Virus binding to cells transfected with plasmid obtained from a positive pool of 50 bacterial transformants (pool 8) following four rounds of FACS enrichment.

(C) Virus binding to cells transfected with plasmid from positive subpool 8-10, containing 5 bacterial transformants.

(D) Virus binding to cells transfected with plasmid from hJAM-encoding clone 8-10-2.

Fab fragments (Fabs) of σ1-specific mAb 9BG5 (Burstin et al., 1982), which binds the T3 σ1 head domain (Chappell et al., 2000), and each of four anti-hJAM mAbs tested (Figure 2E). Control antibodies directed against CD47 (mAb C5/D5) (Parkos et al., 1996) and the human coxsackievirus and adenovirus receptor (hCAR) (J. Bergelson, personal communication), had no effect on reovirus binding. Anti-hJAM mAb J10.4 inhibited T3SA$^-$ binding to NT2 cells in a dose-dependent manner, with a minimal inhibitory concentration between 0.2 and 0.02 μg/ml (Figure 2E). Anti-hJAM mAb J10.4 also significantly inhibited binding of strain T3SA$^+$ to NT2 cells (Figure 2E); however, residual virus binding above background remained. Preincubation of T3SA$^+$ with sialyllactose (SLL) to inhibit binding to cell-surface SA (Barton et al., 2001) abolished T3SA$^+$ binding to NT2 cells treated with mAb J10.4 (Figure 2E). These results indicate that T3SA$^-$ binds to hJAM, while T3SA$^+$ binds to both hJAM and SA on NT2 cells. Together, these results strongly suggest that reovirus binding and infection are dependent on the availability of hJAM to serve as a reovirus receptor.

Transient Transfection of hJAM Renders Murine Erythroleukemia (MEL) Cells Permissive for Infection by T1 and T3 Reovirus Strains

If hJAM functions as a *bona fide* reovirus receptor, then transfection of reovirus-resistant cells with hJAM should permit reovirus infection. To test this prediction, we used

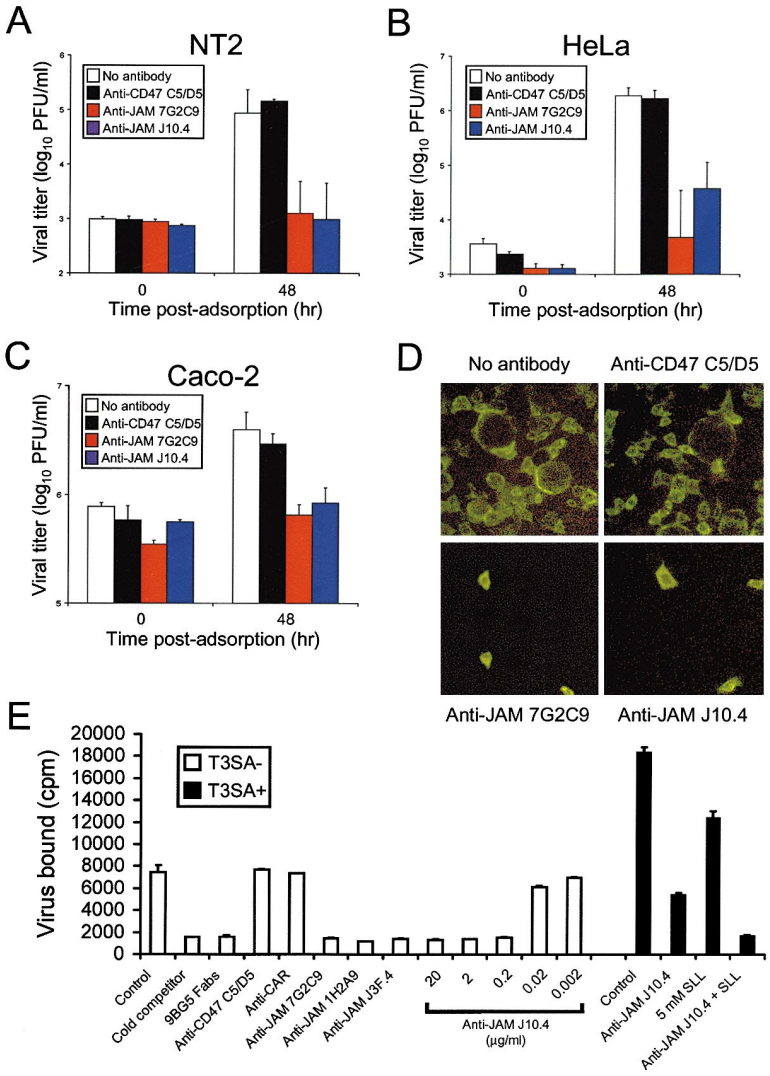

Figure 2. JAM Is Required for Reovirus Binding and Infection of NT2 Cells, HeLa Cells, and Caco-2 Cells

(A–C) Effect of anti-hJAM mAbs on T3SA$^-$ growth in cultured cells. NT2 cells (A), HeLa Cells (B), or Caco-2 cells (C) were incubated in the presence or absence of 20 μg/ml CD47-specific mAb C5/D5 or 1 μg/ml of hJAM-specific mAbs 7G2C9 or J10.4 prior to adsorption with T3SA$^-$ at an MOI of 1 PFU/cell. Antibodies and inocula were removed, cells were incubated for the times shown, and progeny virions were quantitated by plaque assay. Shown are mean viral titers for three experiments. Error bars indicate standard deviations.

(D) Effect of anti-hJAM mAbs on T3SA$^-$ infection of Caco-2 cells. Caco-2 cells were treated with CD47-specific mAb C5/D5 (20 μg/ml) or hJAM-specific mAbs (5 μg/ml) prior to infection with T3SA$^-$ at an MOI of 100 PFU/cell. After incubation for 24 hr, infected cells were visualized by indirect immunofluorescence.

(E) Effect of anti-hJAM mAbs on binding of T3SA$^-$ and T3SA$^+$ to NT2 cells. Cells were untreated as a control, incubated with unlabeled virus as competitor, or pretreated with the mAbs shown prior to adsorption with radioiodinated virions of T3SA$^-$ or T3SA$^+$. Virions also were untreated or pretreated with 5 mM SLL or 9BG5 Fabs (50 μg/ml). Antibodies were used at either 20 μg/ml or the concentrations shown. Cell-associated virus was captured by vacuum filtration and quantitated by liquid scintillation. Error bars indicate the range of values from duplicate experiments.

MEL cells, which are resistant to infection by T1 strains and non-SA-binding T3 strains, but support infection by SA-binding T3 strains (Rubin et al., 1992; Barton et al., 2001). MEL cells were transiently transfected with empty vector or hJAM-encoding plasmid and infected with T3SA$^-$ or T3SA$^+$ (Figure 3A). As expected, T3SA$^+$ but not T3SA$^-$ grew efficiently in vector-transfected cells. In contrast, yields of T3SA$^-$ were 10-fold greater in cells transfected with hJAM than in vector-transfected cells. This result indicates that expression of hJAM in MEL cells rescues infection of non-SA-binding reovirus.

We also tested the effect of transient hJAM expression on growth of prototype reovirus strains T1 Lang (T1L) and T3 Dearing (T3D) in MEL cells (Figure 3B). As anticipated, SA-binding strain T3D grew efficiently in vector- and hJAM-transfected MEL cells. Surprisingly, although T1L did not infect vector-transfected MEL cells, it grew efficiently in hJAM-transfected MEL cells. Given that serotype-dependent differences in reovirus tropism and pathogenesis are thought to be determined by differences in receptor utilization (Sharpe and Fields, 1985), this result was unexpected and suggests that T1 and T3 reovirus strains utilize JAM as a serotype-independent receptor.

To test the hypothesis that JAM is a receptor for both T1 and T3 reovirus, we assessed the capacity of anti-hJAM mAbs to block the binding of T1L and T3D to NT2 cells (Figures 3C and 3D). T1L binding to NT2 cells was abolished by either Fabs of mAb 5C6, which is specific for the T1 σ1 head domain (Chappell et al., 2000), or anti-hJAM mAb J10.4 (Figure 3C). T3D bound much more efficiently than T1L to NT2 cells (Figure 3D), but >60% of T3D binding was inhibited by either 9BG5 Fabs or mAb J10.4 (Figure 3D). Significant binding of T3D remained even in the presence of anti-hJAM mAbs, consistent with the fact that T3D σ1 also can bind SA (Chappell et al., 2000). These results indicate that prototype T1 and T3 reovirus strains recognize hJAM as a receptor on a human neuronal-precursor cell line. However, in addition to recognition of hJAM, strain T3D binds an additional NT2 molecule that is likely to be SA (Figure 2E).

Transient Transfection of hJAM or Murine JAM (mJAM) Renders Chicken Embryo Fibroblast (CEF) Cells Permissive for Infection by T1 and T3 Reovirus Strains

Since MEL cells support growth of some reovirus strains, we thought it possible that transfection of this

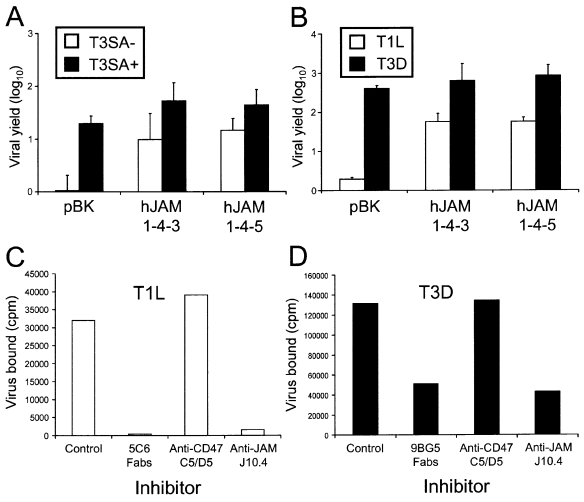

Figure 3. JAM Rescues Infectivity of T1 and T3 Reovirus Strains

(A and B) Effect of JAM expression on growth of T1 and T3 reovirus strains in MEL cells. MEL cells were transiently transfected with control vector (pBK) or hJAM clones 1-4-3 and 1-4-5. Transfected cells were adsorbed with T3SA$^-$, T3SA$^+$, T1L, or T3D at an MOI of 1 PFU/cell and incubated for either 24 (A) or 48 (B) hr. Viral titers were determined by plaque assay. Shown are mean viral yields (titer at 24 or 48 hr divided by titer at 0 hr) for three experiments. Error bars indicate standard deviations.

(C and D) Effect of anti-hJAM mAbs on binding of T1 and T3 reovirus strains to NT2 cells. NT2 cells were incubated with CD47-specific mAb C5/D5 (20 μg/ml) or hJAM-specific mAb J10.4 (2 μg/ml) prior to adsorption with radioiodinated T1L (C) or T3D (D). Virions were pretreated with the inhibitors shown.

cell type with hJAM might upregulate an endogenous receptor. Therefore, we tested the capacity of cDNAs encoding either hJAM or mJAM to confer reovirus infection to CEF cells, which do not support reovirus infection (Figure 4). CEF cells were transiently transfected with hCAR-, hJAM-, or mJAM-encoding plasmids and infected with virions or infectious subvirion particles (ISVPs) of T1L, T3SA$^-$, or T3SA$^+$. ISVPs are reovirus disassembly intermediates generated in vivo in the intestinal lumen or in endocytic vesicles and in vitro by protease treatment (Baer and Dermody, 1997). These particles bind cell-surface receptors but do not require endocytic proteolysis for infection (Baer and Dermody, 1997). ISVPs were used in this experiment since we reasoned that CEF cells might not express endosomal proteases required for mammalian reovirus disassembly. This was found to be the case, since neither control nor JAM-expressing CEF cells supported infection with virions of any reovirus strain (data not shown). However, ISVPs of T1L, T3SA$^-$, and T3SA$^+$ were capable of infecting CEF cells transfected with hJAM or mJAM but not hCAR (Figure 4). These results indicate that the block to reovirus infection in avian cells is rescued by expression of hJAM or mJAM, providing strong evidence that JAM functions as a serotype-independent reovirus receptor in both human and murine hosts. Furthermore, these findings suggest that the attachment and disassembly steps of the reovirus life cycle serve as key determinants of the host-range restriction exhibited by diverse classes of vertebrates.

hJAM Binds Directly to the Reovirus σ1 Head Domain with High Affinity

To exclude the possibility that hJAM indirectly enhances reovirus infection at a post-attachment step, we used surface plasmon resonance (SPR) to determine whether reovirus particles and σ1 protein bind directly to hJAM (Karlsson and Falt, 1997) (Figure 5). For these experiments, we generated a fusion protein consisting of the rabbit Ig Fc domain linked to the extracellular domain of hJAM (Fc-hJAM). A fusion of the rabbit Fc with hCAR (Fc-hCAR) was used as a control for nonspecific interactions (Bergelson et al., 1997). Fc-hJAM and Fc-hCAR were conjugated to SPR sensor chips, and purified virions of T3SA$^-$, T3SA$^+$, T1L, or T3D were injected across the Fc-hJAM and Fc-hCAR flow cells (Figure 5A). Virions of all four strains displayed a time-dependent increase in binding to Fc-hJAM, and this binding was stable during buffer wash. Virions did not bind to Fc-hCAR, and preinjection of anti-hJAM mAbs blocked virus binding to hJAM, indicating that binding was specific for the hJAM extracellular domain (Figure 5A and data not shown).

To determine whether reovirus binding to hJAM is mediated by σ1, T3SA$^+$ virions were incubated with

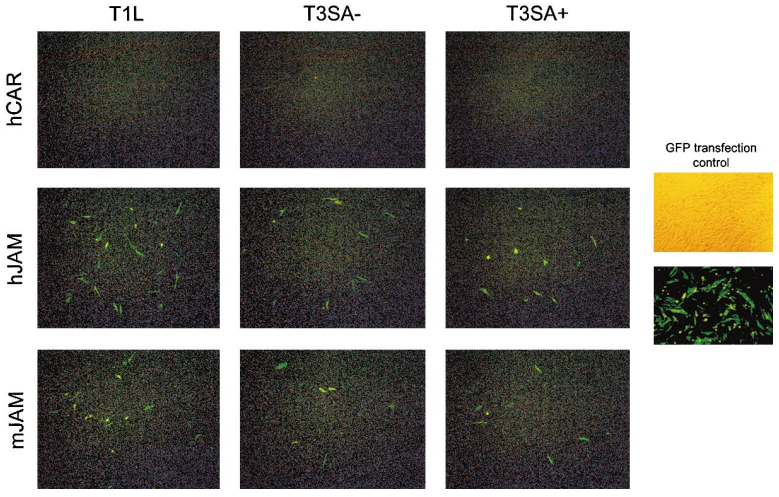

Figure 4. Transfection of CEF Cells with hJAM or mJAM Enables Infection by T1 and T3 Reovirus

CEF cells were transiently transfected with plasmid encoding hCAR, hJAM, or mJAM. Transfected CEF cells were adsorbed with ISVPs of T1L, T3SA$^-$, or T3SA$^+$ at an MOI of 10 PFU/cell. Reovirus antigen was detected by indirect immunofluorescence 20 hr postinfection. Parallel transfections were performed using pEGFP-N1 as an indicator of transfection efficiency.

specific inhibitors of either the σ1 SA binding domain (SLL) or the σ1 head RBD (9BG5 Fabs) (Figure 5B). Binding of T3SA$^+$ to Fc-hJAM was not inhibited by SLL, indicating that binding to SA on hJAM glycosylation chains is not required for this interaction. In contrast, preincubation of T3SA$^+$ virions with 9BG5 Fabs, but not control 5C6 Fabs, substantially reduced T3SA$^+$ binding to Fc-hJAM, suggesting that this binding is mediated by the σ1 head.

To investigate whether hJAM interacts directly with σ1, we tested the capacity of recombinant σ1 to bind Fc-hJAM (Figure 5C). Purified T3D σ1 (Chappell et al., 2000) bound specifically, saturably, and reversibly to Fc-hJAM. Kinetic analysis of the σ1-Fc-hJAM interaction assuming 1:1 stoichiometry indicated a K_D of approximately 9×10^{-8} M (Karlsson and Falt, 1997). SLL had no effect on the binding of σ1 to Fc-hJAM, confirming that this interaction does not require SA (data not shown).

To confirm that the σ1 head mediates binding to hJAM, a proteolytically derived fragment of σ1 containing only the σ1 head was injected over the Fc-hJAM surface (Figure 5C). The σ1 head domain bound specifically to Fc-hJAM with a K_D of 6×10^{-8} M, which approximates that of full-length σ1. These results indicate that the head domain of reovirus attachment protein σ1 binds with high affinity directly to the extracellular domain of hJAM.

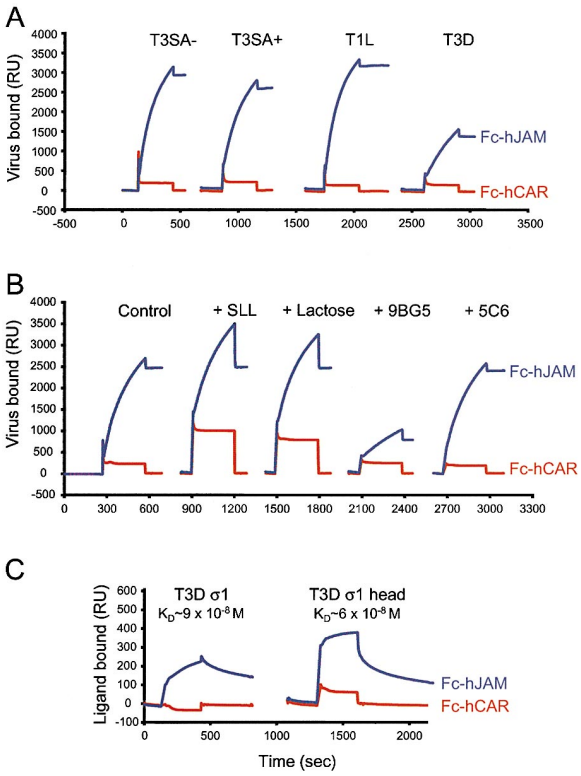

Figure 5. Quantitation of σ1 Binding Affinity to hJAM Using Surface Plasmon Resonance

(A) T1 and T3 reovirus strains interact directly with hJAM. Virions of T3SA$^-$, T3SA$^+$, T1L, or T3D were injected over biosensor surfaces coated with either Fc-hJAM or Fc-hCAR fusion proteins. Binding in response units was measured over time.

(B) T3SA$^+$ binding to hJAM is not dependent on SA. Binding of T3SA$^+$ to Fc-hJAM and Fc-hCAR on a biosensor was assessed in the presence of SLL, lactose (control), or Fabs of 9BG5 or 5C6 (control).

(C) The σ1 head domain binds to hJAM. Purified T3D σ1 or σ1 head domain were injected over a biosensor coated with Fc-hJAM or Fc-hCAR. Calculated affinities for binding to Fc-hJAM, expressed as apparent K_D, are shown.

Reovirus–JAM Interactions Are Required for Activation of NF-κB and Induction of Apoptosis during Reovirus Infection

Reovirus infection of cultured cells leads to NF-κB activation, which is required for reovirus-induced apoptosis (Connolly et al., 2000). To determine whether engagement of JAM by σ1 triggers these cellular responses, we tested the capacity of T3SA$^+$ to replicate, activate NF-κB, and induce apoptosis in the presence of anti-hJAM mAbs. The presence of residual binding of T3SA$^+$ and T3D to NT2 cells preincubated with anti-hJAM mAbs (Figures 2E and 3D) suggested that reovirus can bind SA in the absence of σ1–JAM interactions. To assess whether binding to SA alone could mediate reovirus entry, HeLa cells were incubated in the presence or absence of anti-hJAM mAb J10.4 prior to adsorption with 100 PFU/cell of T3SA$^+$. Although infection of T3SA$^-$ is substantially inhibited in HeLa cells incubated with mAb J10.4 (Figure 2B), growth of T3SA$^+$ was only minimally reduced (less than 2-fold at 48 hr) by hJAM blockade (Figure 6A). Consistent with this finding, we also observed ~50% fewer reovirus-antigen-positive cells following hJAM blockade and adsorption with T3SA$^+$ at an MOI of 1 PFU/cell (data not shown). These results suggest that cell-surface SA can serve as a functional receptor when hJAM is absent or inaccessible.

Since T3SA$^+$ can infect HeLa cells via an hJAM-independent pathway, it was possible to determine whether σ1–hJAM interactions modulate the efficiency of reovirus-induced NF-κB activation. We found that T3SA$^+$ induced strong activation of NF-κB in untreated cells, but this activation was abolished in cells treated with anti-hJAM mAb J10.4 (Figure 6B). Since NF-κB activation is required for induction of apoptosis in reovirus-infected HeLa cells (Connolly et al., 2000), we predicted that blockade of NF-κB activation by mAb J10.4 would correlate with decreased apoptosis. In untreated cells, T3SA$^+$ elicited high levels of apoptosis (Figure 6C). However, incubation of cells with mAb J10.4 abolished the capacity of T3SA$^+$ to induce this response. Importantly, treatment of HeLa cells with mAb J10.4 had no effect on the capacity of TNF-α to activate NF-κB and induce apoptosis, indicating that neither the NF-κB signaling machinery nor the apoptotic response is inhibited by anti-hJAM mAb J10.4 (Figures 6B and 6C). These results demonstrate that while SA can mediate entry of SA-binding reovirus strains, reovirus-induced NF-κB activation and resultant cell death require virus-induced signaling events mediated by the interaction of σ1 with JAM.

Discussion

Results presented in this report demonstrate that JAM satisfies all requisite criteria of a functional reovirus re-

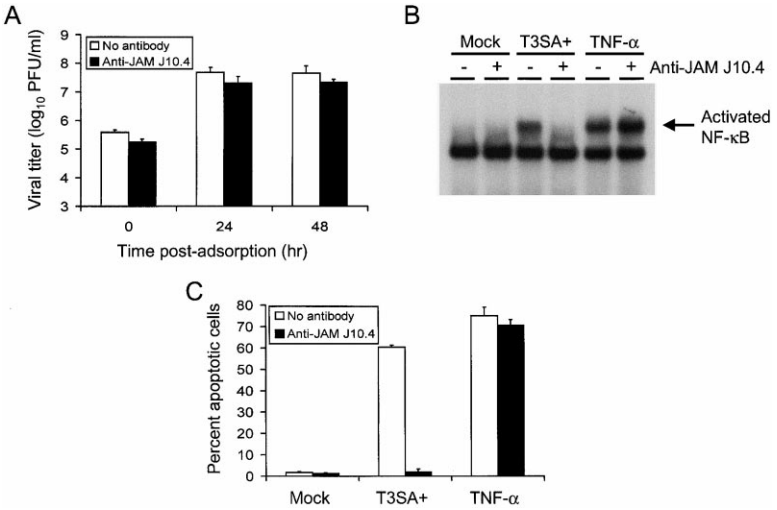

Figure 6. Anti-hJAM mAbs Inhibit NF-κB Activation and Apoptosis Induction by Reovirus T3SA$^+$

(A) Effect of anti-hJAM mAb J10.4 on growth of T3SA$^+$ in HeLa cells. HeLa cells were incubated with PBS or 5 μg/ml mAb J10.4 prior to adsorption with T3SA$^+$ at an MOI of 100 PFU/cell. Shown are mean viral titers for three experiments. Error bars indicate standard deviations.

(B) Effect of anti-hJAM mAb J10.4 on NF-κB activation induced by T3SA$^+$ and TNF-α. HeLa cells were incubated with PBS or 5 μg/ml mAb J10.4 prior to adsorption with T3SA$^+$ (100 PFU/cell) or treatment with TNF-α (20 ng/ml). Mock-infected cells are shown as a control. After incubation for either 1 (TNF-α) or 10 (T3SA$^+$) hr, NF-κB complexes in nuclear extracts were detected by EMSA.

(C) Effect of anti-hJAM mAb J10.4 on apoptosis induced by T3SA$^+$ and TNF-α. HeLa cells were incubated with PBS or 5 μg/ml mAb J10.4 prior to adsorption with T3SA$^+$ (100 PFU/cell) or treatment with TNF-α (20 ng/ml). Mock-infected cells are shown as a control. After incubation for either 24 (TNF-α) or 48 (T3SA$^+$) hr, cells were stained with acridine orange. Shown is the mean percentage of cells undergoing apoptosis for three experiments. Error bars indicate standard deviations.

ceptor. First, transfection of COS-7 cells with hJAM confers binding of reovirus T3SA$^-$. Second, blockade of hJAM on the surface of NT2 cells, HeLa cells, or Caco-2 cells abolishes T3SA$^-$ binding and growth. Third, binding of prototype reovirus strains T1L and T3D to NT2 cells is blocked by hJAM-specific mAbs. Fourth, transfection of murine and avian cells with hJAM rescues reovirus infection in a serotype-independent manner. Finally, and most conclusively, the biological effects of hJAM on reovirus infection correlate with a direct, SA-independent, high-affinity interaction between hJAM and the head domain of reovirus attachment protein σ1.

JAM is a type I transmembrane protein with two extracellular Ig domains and a short cytoplasmic tail (Martin-Padura et al., 1998; Liu et al., 2000). JAM is an important component of TJs between endothelial and epithelial cells, and it may function to organize TJ formation by interaction with other TJ proteins and the cytoskeleton (Bazzoni et al., 2000; Ebnet et al., 2000). JAM also may influence the migration of leukocytes across endothelial and epithelial barriers during the course of an inflammatory response (Del Maschio et al., 1999; Lechner et al., 2000). JAM is highly conserved among mammals, with human, murine, bovine, and rat JAM displaying ~70% amino acid identity (Martin-Padura et al., 1998; Liu et al., 2000). Given the broad host range of mammalian reovirus (Tyler and Fields, 1996), it is not surprising that a reovirus receptor would display a high degree of sequence conservation.

JAM is expressed on many cell types that are known targets for reovirus infection in vivo, including intestinal epithelium, bile duct epithelium, lung epithelium, leukocytes, and CNS endothelial cells (Martin-Padura et al., 1998; Williams et al., 1999; Liu et al., 2000). These observations are consistent with the extensive and overlapping tissue distribution of T1 and T3 reovirus infections in mice, particularly within the intestine (Tyler and Fields, 1996). Cell-surface JAM is localized to the subapical surface of polarized epithelial TJs (Martin-Padura et al., 1998; Liu et al., 2000). Reovirus gains access to the basolateral surface of intestinal cells by transport through microfold cells (Wolf et al., 1981), which would allow virus exposure to the area of highest JAM expression. It is also possible that transient disruptions of the TJ barrier, such as those that occur during migration of immune and inflammatory cells, permit reovirus access to JAM from the intestinal lumen. Such micro-disruptions of TJ integrity facilitate infection by other pathogens, including *Yersinia* (McCormick et al., 1997) and *Salmonella* (Jensen et al., 1998).

Since the discovery that differences in the tropism of T1 and T3 reovirus for specific cells in the CNS segregate with the σ1-encoding S1 gene, it has been hypothesized that T1 and T3 strains bind to distinct receptors expressed on ependymal cells and neurons, respectively (Weiner et al., 1980; Sharpe and Fields, 1985). However, JAM confers infection by both T1 and T3 reovirus strains, suggesting that the interaction between JAM and the σ1 head domain is not the critical determinant of serotype-dependent differences in reovirus CNS tropism. Instead, we think it possible that differences in the cell-surface carbohydrates bound by T1 and T3 σ1 proteins influence viral tropism in the murine nervous system. Accordingly, neural polysialic acid (Rutishauser and Landmesser, 1996) may permit infection by T3 strains, as was observed for T3SA$^+$ infection of HeLa cells. Since T1 strains do not bind SA (Chappell et al., 2000), these strains would be incapable of utilizing this pathway and might even be repelled by the negatively-charged SA moieties on the neural surface. Alternatively, it is possible that proteins with homology to JAM display serotype-specific interactions with σ1.

T3 reovirus infects neurons and causes encephalitis in neonatal mice, but mice rapidly become resistant to reovirus disease during the first few weeks of life (Tardieu et al., 1983). As embryonic neuronal differentiation progresses, the neuroepithelium down-regulates integral TJ proteins and loses the capacity to form TJs

(Aaku-Saraste et al., 1996). JAM is not highly expressed on adult mouse neurons (Martin-Padura et al., 1998); however, its expression in the developing murine nervous system has not been rigorously examined. It is possible that JAM is expressed at birth and then declines as neonatal CNS remodeling is completed, thereby providing a mechanistic explanation for the age-restriction of reovirus encephalitis.

The capacity of reovirus to interact with the TJ via JAM may have important consequences for the pathology of reovirus infection. Regulation of the TJ is critical for maintenance of epithelial and endothelial barriers (Bazzoni et al., 1999). Anti-hJAM mAbs prevent the reorganization of disrupted TJs in cultured intestinal epithelial cells (Liu et al., 2000) and promote TJ breakdown of the endothelium lining CNS blood vessels, resulting in enhanced disease in response to bacterial or viral infections (Lechner et al., 2000). If reovirus–JAM interactions lead to a similar destabilization of TJs in CNS endothelium, this might promote breakdown of the blood-brain barrier, permitting cerebral edema and neural inflammation, conditions evident in reovirus encephalitis (Tyler and Fields, 1996). In addition, reovirus-induced TJ dysregulation within the murine intestinal epithelium might promote diarrhea, thereby enhancing viral shedding and transmission. A similar mechanism has been proposed for the pathogenesis of diarrhea induced by rotavirus (Obert et al., 2000).

Our finding that σ1–JAM interactions are required for NF-κB activation and apoptosis underscores the importance of the attachment step in modulating the cellular consequences of reovirus infection. Although T3SA$^+$ is capable of efficient infection via SA-mediated attachment, this route of infection seems to bypass the cellular signaling events culminating in apoptosis, suggesting that viral replication is not sufficient to trigger this response. Our findings demonstrate that reovirus binding to JAM is required for induction of the apoptotic response; however, σ1–JAM interactions are not the sole determinant of virus-induced apoptosis. T3SA$^+$ is much more efficient than T3SA$^-$ at inducing NF-κB-dependent apoptosis in HeLa cells (J. L. C. et al., submitted), despite the fact that both strains bind JAM on this cell type. We propose a model in which a virus-cell synapse formed by multivalent interactions of σ1 with both JAM and SA surpasses a critical cellular activation threshold required for NF-κB activation and apoptosis (Figure 7). This model has similarities to mechanisms of lymphocyte activation in which lymphocyte receptors must be engaged by both antigen and accessory molecules to initiate effector functions (Grakoui et al., 1999). JAM appears to be the only receptor on some cell types for non-SA-binding reovirus strains. However, the binding of these strains to JAM is not sufficient to trigger NF-κB activation (Figure 7A). In contrast, the simultaneous ligation of JAM and SA moieties on JAM by SA-binding strains may alter the conformation or oligomeric nature of JAM, thereby triggering signaling events subsequent to virus binding (Figure 7B). Alternatively, SA-binding strains might bind JAM and SA residues on other cellular proteins, and the juxtaposition of JAM with these proteins may activate signaling cascades that culminate in apoptotic cell death (Figure 7C). In support of the observed coupling of JAM and NF-κB, JAM exists in a complex

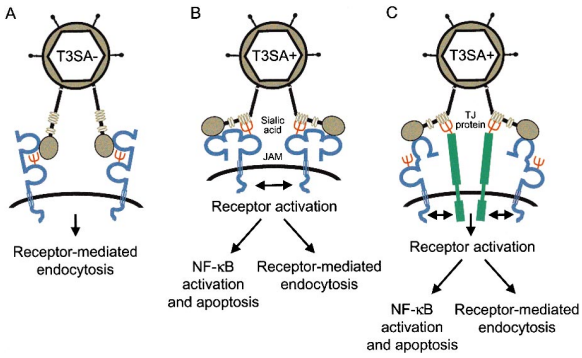

Figure 7. Models of Reovirus–JAM Interactions at the Cell Surface

(A) Non-SA-binding reovirus strains like T3SA$^-$ bind JAM as a receptor. Binding of these strains to JAM mediates virus internalization but does not induce signals leading to activation of NF-κB.

(B) SA-binding reovirus strains like T3SA$^+$ bind both JAM and SA. The membrane-proximal Ig domain of hJAM contains two N-linked glycosylation sites (Liu et al., 2000), one of which is schematically indicated. The dual ligation of JAM and JAM-bound SA is proposed to induce JAM cross-linking or conformational alterations leading to initiation of signals required for NF-κB activation and apoptosis.

(C) SA-binding reovirus strains also may activate intracellular signaling pathways by the simultaneous crosslinking of JAM and other sialyllated cell-surface proteins.

of TJ proteins that includes the Ras-interacting protein AF-6 (Bazzoni et al., 2000; Ebnet et al., 2000). Importantly, Ras-mediated pathways have been implicated in activation of NF-κB (Anrather et al., 1999; Norris and Baldwin, 1999; Romashkova and Makarov, 1999). Since infection of newborn mice with T3 reovirus at a dose that leads to lethal encephalitis also induces apoptosis in the CNS (Oberhaus et al., 1997), our findings raise the possibility that reovirus–JAM interactions dictate strain-specific pathogenic phenotypes as a consequence of intracellular signaling in addition to determining viral tropism.

It is interesting to speculate why reoviruses would utilize a receptor that triggers apoptotic death of the infected cell. Although many viruses induce apoptosis of host cells, only a select group encodes proteins that actively inhibit this process. For these viruses, it is likely that apoptosis must be blocked for a period sufficient to assemble viable progeny (Roulston et al., 1999). Reovirus replication requires ~16 hr; however, the majority of reovirus-infected cells remain viable for up to 48 hr post-infection (Connolly et al., 2000). This interval would allow for multiple rounds of viral replication prior to cell death. Thus, there may be no selective advantage for reovirus to inhibit the host cell apoptotic response. In fact, there is increasing evidence to suggest that some viruses utilize apoptosis as a means to evade the host immune response by minimizing tissue inflammation and permitting viral spread within apoptotic bodies (Teodoro and Branton, 1997; O'Brien, 1998). Identification of JAM as a signaling molecule required for reovirus-induced apoptosis will permit dissection of the underlying mechanisms and pathologic significance of apoptosis in response to viral infection.

Although a number of signaling molecules concentrate at the cytoplasmic face of the TJ, their roles in

transducing signals from the environment to the nucleus are unclear (Clarke et al., 2000; Hopkins et al., 2000). We provide the first demonstration that JAM and the TJ are involved in a discrete outside-in nuclear signaling pathway. NF-κB regulates the transcription of many inflammatory cytokines involved in the activation and attraction of immune effector cells (Pahl, 1999). The linkage of JAM to NF-κB has important implications for the role of the TJ in responding to pathogens and suggests that the TJ can serve as an environmental sensor capable of responding to viral infection by triggering apoptosis. The discovery of JAM as a reovirus receptor expands our knowledge of virus-receptor biology and highlights a potential role for the TJ in regulating both inflammatory responses and cell death.

Experimental Procedures

Cells, Viruses, and Antibodies

L cells, MEL cells, and HeLa cells were maintained as described (Barton et al., 2001). NTERA-2 (NT2) and Caco-2 cells were obtained from the American Type Culture Collection and maintained in monolayer culture as for HeLa cells. CEF cells were derived from day 10 fertilized chicken eggs and maintained as described (Brown et al., 1999).

Reovirus strains T1L and T3D are laboratory stocks. Isogenic σ1-point mutants T3/C44-SA$^-$ and T3/C44-SA$^+$, abbreviated in this manuscript as T3SA$^-$ and T3SA$^+$, respectively, were generated as described (Barton et al., 2001). Viral titer was determined by plaque assay on L cells (Virgin et al., 1988). Purified virions were prepared and quantitated as described (Furlong et al., 1988). ISVPs were generated as described (Baer and Dermody, 1997). Purified T3SA$^-$ (1×10^{13}/ml in carbonate-bicarbonate buffer [pH 9] [Sigma-Aldrich, St. Louis, MO]) was fluoresceinated by incubation in 50 μg/ml FITC (Pierce, Rockford, IL) for 1 hr at room temperature.

Murine mAbs 9BG5 (Burstin et al., 1982) and 5C6 (Virgin et al., 1991) were purified from hybridoma supernatants (Cell Culture Center, Minneapolis, MN), and Fabs of each were prepared using the Immunopure Fab system (Pierce). Anti-hJAM murine mAbs J10.4, 7G2C9, J3F.4, and 1H2A9 (all IgG1) (Williams et al., 1999; Liu et al., 2000) and CD47-specific mAb C5/D5 (IgG1) (Parkos et al., 1996) were purified from ascites by protein A affinity. Rabbit hCAR-specific antiserum was provided by Dr. Jeffrey Bergelson (University of Pennsylvania).

Expression Cloning of hJAM

COS-7 cells were transfected with an NT2 cDNA library (Stratagene, La Jolla, CA) as described (Aruffo and Seed, 1987). After 48 hr incubation, cells were detached from plates by incubation with 2 mM EDTA/PBS at 37°C. Detached cells (1×10^6) were resuspended in 150 μl PBS containing 1×10^{11} FITC-conjugated T3SA$^-$ particles and incubated on ice for 1 hr. Cells were washed twice with PBS, viable cells were analyzed by FACS, and the 0.5% most fluorescent cells were collected using a FACStar Plus (Becton, Dickinson and Co., Franklin Lakes, NJ). Plasmid was rescued from sorted cells (Hirt, 1967), amplified in bacteria, and used in three subsequent rounds of FACS enrichment. Individual bacterial colonies obtained from the quaternary sort were grouped into pools of 50 colonies. Plasmid prepared from pools was used to transfect COS-7 cells. Positive pools were defined as those conferring a greater than 2% increase in the number of maximally-fluorescent cells as compared to cells transfected with cDNA library. Positive pools were subdivided into sub-pools of 5 colonies, and plasmid from sub-pools was tested for the capacity to confer enhanced FITC-T3SA$^-$ binding to transfected COS-7 cells. Individual clones from positive sub-pools were similarly screened. This process yielded four clones that conferred T3SA$^-$ binding to all transfected COS-7 cells (1-4-3, 1-4-5, 8-10-2, and 8-10-3). Automated sequencing revealed that each clone encoded hJAM (Liu et al., 2000).

Assessment of Virus Growth

Cells ($2–5 \times 10^5$) were incubated in PBS or PBS containing various concentrations of mAbs C5/D5, 7G2C9, or J10.4 at room temperature for 1 hr. Virus was adsorbed to antibody-treated cells at an MOI of 1 or 100 PFU/cell and incubated at room temperature for 1 hr. Inocula were removed, cells were washed, and complete medium was added. Cells were incubated at 37°C for various intervals, and viral titers in lysates were determined by plaque assay.

Fluorescent-Focus Assay of Viral Infection

Virus was adsorbed to confluent monolayers as for growth experiments. Following incubation at 37°C for 20 hr, cells were fixed with 1 ml of methanol at −20°C for 30 min. Infected cells were identified by indirect immunofluorescence using rabbit anti-reovirus sera as described (Barton et al., 2001).

Virus Radioligand Binding Assays

Purified virions ($2–4 \times 10^{13}$/ml in Dulbecco's PBS [Gibco-BRL, Grand Island, NY]) were iodinated and used for binding assays as described (Barton et al., 2001). Iodinated virus was added to cells (1×10^6) and incubated at room temperature for 3 hr. Cell-associated virus was captured by vacuum filtration and quantitated by liquid scintillation. For experiments assessing the effect of SLL or Fabs on virus attachment, iodinated virions were preincubated with each reagent at 37°C for 30 min. Antibodies against cell-surface proteins were preincubated with cells at 37°C for 30 min prior to adsorption of radiolabeled virus.

Cloning of mJAM

mJAM was cloned by PCR amplification from Quickclone cDNA derived from poly-A mRNA of 7-day murine embryos (Clontech, Palo Alto, CA). cDNA (4 ng) was subjected to PCR with primers (0.2 μM) specific for the reported mJAM sequence appended at the 5′ and 3′ termini with XbaI and SpeI restriction sites, respectively (5′-CCT ACTAGTGGATTGTAACTGTAATGGGCA-3′ and 5′-CCTTCTAGAGC CGCAGCAGGTCACACCAGG-3′) (Martin-Padura et al., 1998). PCR product was purified using DNAzol (Molecular Research Center, Cincinnati, OH), treated with XbaI and SpeI, and ligated into the XbaI and SpeI sites of alkaline phosphatase-treated pBK-CMV (Stratagene). Fidelity of PCR amplification and cloning was confirmed by automated sequencing.

Transient Transfection and Reovirus Infection of MEL Cells

MEL cells (1×10^7) were transiently transfected with 40 μg each of pEGFP-N1 (Clontech) (transfection control), pBK-CMV (negative control), hJAM 1-4-3, or hJAM 1-4-5 by electroporation. Transfection efficiency was ~10%. After 48 hr incubation, 2×10^5 cells were adsorbed with T3SA$^-$, T3SA$^+$, T1L, or T3D at an MOI of 1 PFU/cell in a total volume of 150 μl. Adsorptions were terminated after incubation at room temperature for 1 hr by washing in PBS. Cells were incubated at 37°C in 1 ml of culture medium for various intervals, and viral titers in lysates were determined by plaque assay.

Transient Transfection and Reovirus Infection of CEF Cells

Passage-five CEF cells (50%–75% confluence) were transfected with 0.4 μg of plasmid encoding hCAR (in pCDNA 3.1), hJAM, or mJAM using Lipofectamine Plus (Gibco-BRL). Transfected CEF cells were infected with virions or ISVPs of T3SA$^-$, T3SA$^+$, or T1L at an MOI of 10 PFU per cell and processed for fluorescent-focus assay.

Generation and Purification of Fc-hJAM Fusion Protein

The hJAM extracellular domain was amplified by PCR from 1 μg of hJAM clone 1-4-5 using hJAM-specific primers (0.2 μg) appended with HindIII and BamHI restriction sites at the 5′ and 3′ termini, respectively (5′-TAGCAAGCTTCCTGATCGCGATG-3′ and 5′-TACG GGATCCATTCCGCTCCAC-3′). PCR product and Fc-hCAR-pCDNA 3.1 (Bergelson et al., 1997) were subjected to restriction digest with HindIII and BamHI, resulting in excision of hCAR-encoding sequences from the plasmid vector. Digestion products were purified by agarose gel electrophoresis, and the hJAM PCR product was ligated into the digested Fc-pCDNA plasmid. Fidelity of PCR amplification and cloning was confirmed by automated sequencing.

The Fc-hJAM and Fc-hCAR plasmid constructs were amplified in

bacteria and used in DEAE/dextran transfections of COS-7 cells (Aruffo and Seed, 1987). After 72 hr incubation, supernatants were harvested, and the soluble Fc-fusion proteins were purified by protein A affinity.

Purification of T3D σ1

T3D-derived σ1 protein was generated, expressed, and purified as described (Chappell et al., 2000). The σ1 head domain was purified following trypsin digestion of σ1 deletion mutant 3-Δ-3-3-3 (Chappell et al., 2000). The 3-Δ-3-3-3 mutant was concentrated to 0.3 mg/ml in PBS using a centrifugal concentrator (Millipore, Bedford, MA) and incubated at 4°C for 1 hr with 10% (v/v) PBS-washed trypsin-agarose beads (Sigma-Aldrich). Trypsin beads were pelleted and the supernatant was removed. Stable fragments corresponding to the σ1 head (25 kDa) and tail (14 kDa) are produced by this treatment (Chappell et al., 2000). Trypsin-treated 3-Δ-3-3-3 σ1 was incubated in an equal volume of Sepharose Q (Amersham Pharmacia) in 20 mM Tris-HCl (pH 8.1) at room temperature for 1 hr. Sepharose Q beads were pelleted, and the σ1 head fragment was eluted from beads using 0.1 M sodium acetate and 0.5 M NaCl (pH 4.0). Eluted fragments were visualized by SDS-PAGE and found to include the 25 kDa σ1 head fragment free of contaminating 3-Δ-3-3-3 σ1 or σ1 tail fragment.

Assessment of Virus-hJAM and σ1–hJAM Interactions Using SPR

Fc-hJAM and Fc-hCAR at a concentration of 0.2–0.4 mg/ml in PBS were biotinylated by incubation with a 10-fold molar excess of sulfo-NHS-LC-biotin (Pierce) at room temperature for 1 hr. A BIAcore CM5 chip (Pharmacia Biosensor AB, Uppsala, Sweden) was coated with 400 μg/ml streptavidin (Sigma-Aldrich) by amine coupling. Biotinylated fusion proteins were injected at a concentration of 5 to 50 μg/ml in HEPES-buffered saline (HBS) (pH 7.2) across duplicate flow cells of a streptavidin-coated chip at 10 μl/min using a BIAcore 2000 (Pharmacia Biosensor AB). Sensor-chip flow cells were coated with 2000 RU of each protein. Purified reovirus virions (5×10^{12} particles/ml) were injected across the conjugated chip surfaces at 10 μl/min. Following virus binding, chip surfaces were regenerated with a 1 min pulse of a 1:1 mixture of HBS and Immunopure Gentle Ag/Ab Elution Buffer (Pierce). Virions also were preincubated with 10 mM SLL, 10 mM lactose (Sigma-Aldrich), or 50 μg/ml Fabs of 9BG5 or 5C6 at room temperature for a minimum of 10 min prior to injection.

Purified σ1 and σ1 head fragments (80 μg/ml) were injected across flow cells as for virions. Affinity constants for σ1 binding to Fc-hJAM were determined using separate k_{on} and k_{off} nonlinear regression with BIAevaluation 3.0 software (Pharmacia Biosensor AB), assuming a 1:1 Langmuir binding model (Karlsson and Falt, 1997). Molar concentrations of σ1 constructs were determined assuming that σ1 exists as a homotrimer (Leone et al., 1992).

Electrophoretic Mobility Shift Assay (EMSA)

Cells (5×10^6) were either untreated or treated with 5 μg/ml mAb J10.4 in PBS. After incubation at 37°C for 30 min, cells were adsorbed with $T3SA^+$ at an MOI of 100 PFU/cell or treated with 20 ng/ml human recombinant TNF-α (Sigma-Aldrich). After incubation at 37°C for either 10 ($T3SA^+$) or 1 (TNF-α) hr, nuclear extracts were prepared and assayed for NF-κB activation by EMSA using a ^{32}P-labeled oligonucleotide consisting of the NF-κB consensus binding sequence (Santa Cruz Biotechnology, Santa Cruz, CA) (Connolly et al., 2000).

Quantitation of Apoptosis by Acridine Orange Staining

Cells (5×10^4) were untreated or treated with 5 μg/ml anti-hJAM mAb J10.4 in PBS. After incubation at 37°C for 30 min, cells were adsorbed with $T3SA^+$ at an MOI of 100 PFU/cell or treated with 20 ng/ml TNF-α and 10 μg/ml cycloheximide (ICN, Aurora, OH). After incubation at 37°C for 48 ($T3SA^+$) or 24 (TNF-α) hr, the percentage of apoptotic cells was determined using acridine orange staining (Tyler et al., 1995). For each experiment, 200 to 300 cells were counted.

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